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13. ABSTRACT *(Maximum 200*

Our research has focused this year on the signal transduction pathways by which tumor necrosis factor (TNF) activates the stress-activated protein kinases (SAPKs, also called Jun N-terminal kinases, JNKs) and the p38s. TNF can promote the apoptotic death of breast cancer cells and exploitation of the proapoptotic signaling pathways mediated by TNF could prove efficacious in the treatment of breast cancer. Alternatively, inasmuch as the SAPK and p38 pathways are activated by many agents commonly used to treat cancer (ionizing radiation, genotoxins such as cisplatin and etoposide, for example) inhibition of these stress-regulated signaling pathways could reduce the disfiguring side effects of cancer treatments. Our results indicate that the TNF receptor associated factor 2 (TRAF2) protein mediates SAPK and p38 activation by physically associating with two proximal protein kinases: germinal center kinase (GCK) and receptor interacting protein (RIP). GCK and RIP, in turn, associate with mitogen-activated protein kinase-kinase-kinases (MAP3Ks) upstream of the SAPKs and p38s. We have succeeded in generating an MCF7 breast carcinoma cell line which stably expresses GCK under the control of a tetracyclin-inducible promoter. These cells will be used in studies of the biology of GCK signaling.

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FOREWORD

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1. INTRODUCTION

1.1 Background Pertinent to Previous and Ongoing Work

1.1.1 *General Considerations*

Cells respond to extracellular stimuli through the recruitment of signal transduction pathways. These pathways receive the external signal and act to mount a response appropriate to this signal. Pathologic gain of function mutations in growth promoting signaling pathways have been implicated in the pathogenesis of breast cancer. Conversely, exploitation of stress-regulated signaling pathways which promote cell cycle arrest or cell death (apoptosis) could be a useful strategy in combating cancer. Our studies focus on the latter phenomena and, accordingly, we have characterized two signal transduction pathways recruited by stressful and inflammatory stimuli, the stress-activated protein kinase (SAPK, also called Jun N-terminal kinase, JNK) pathway and the p38 pathway.

1.1.2 *ERK/MAPK pathways in eukaryotic cells: an emerging paradigm, the core signaling module*

The SAPKs and p38s are members of the extracellular signal-regulated kinase (ERK)/mitogen-activated protein kinase (MAPK) family of protein Ser/Thr kinases. Signal transduction mechanisms composed of a core of protein Ser/Thr kinases culminating in ERK/MAPK activation have been widely conserved in eukaryotic evolution. All eukaryotic cells possess multiple ERK/MAPK pathways which are poised to respond preferentially to distinct extracellular inputs. The existence of multiple parallel pathways allows a cell to respond simultaneously to different classes of stimuli [1,2].

At the heart of all ERK/MAPK pathways is a central, three tiered core module of protein kinases wherein the ERKs/MAPKs are activated by concomitant Tyr and Thr phosphorylation catalyzed by members of the MAPK/ERK kinase (MEK) family. MEKs, in turn, are activated by Ser/Thr phosphorylation catalyzed by several protein kinase families collectively termed MAPK kinase-kinases (MAP3Ks) [1,2]. A diverse variety of molecular species has been implicated in the regulation of MAP3K → MEK → ERK/MAPK core pathways. In mammals, these include small GTPases of the Ras superfamily as well as protein kinases of the Ste20 and Sps1 families, and adaptor proteins which couple to cytokine receptors [1,2]. The best characterized mammalian ERK/MAPK pathway is the Ras-MAPK pathway wherein mitogen receptors, such as those for EGF or insulin, recruit Ras which, in turn elicits activation of the MAP3K Raf-1. Raf-1 activates the mitogenic MEKs MEK1 and MEK2 which then activate the p42 and p44 MAPKs [3]. Whereas this mitogenic ERK/MAPK pathway has been elucidated in good detail; in general, the regulation of most mammalian MAP3Ks and, by extension, most ERK/MAPK core signaling modules is poorly understood.

1.1.3 *The SAPK and p38 pathways: mammalian ERK/MAPK pathways activated by stress and inflammatory cytokines*

The SAPKs and the p38s are two recently described ERK/MAPK subfamilies activated preferentially by environmental stresses. The SAPKs and p38s are poorly activated by mitogens such as EGF or insulin [1,4-9]. Of particular importance, the SAPKs and p38s are major targets of two inflammatory cytokines: TNF and IL1 [1,4-9]. In response to stressful and inflammatory stimuli, the SAPKs and p38s can regulate gene expression through the direct phosphorylation and activation of transcription factors. The SAPKs and p38s are crucial elements in the program of TNF-induced gene expression, and therefore to the physiology of TNF, in their capacity as the dominant kinases responsible for activation of the activator protein-1 (AP-1) transcription factor [4,5,10].

AP-1 typically consists of a heterodimer of c-Jun and a member of the c-Fos or activating transcription factor (ATF) families [10]. AP-1 dimers bind to a *cis* acting element, the TPA response element (TRE) in the promoters of stress and mitogen-regulated genes. Activation of AP-1 *trans* activating activity and DNA binding, either through phosphorylation of, or elevations in the levels of AP-1 constituents, in turn, recruits the transcriptional machinery [10]. Both the SAPKs and p38s can regulate AP-1 at several levels. SAPK

phosphorylation of c-Jun within the *trans* activation domain (Ser 63 and 73) correlates well with elevated c-Jun/AP-1 *trans* activating activity [4,5,10]. SAPK phosphorylation of the transcription factor Elk1 at Ser 383 and 389 activates ternary complex factor function which, through its association with the serum response factor, participates in the induction of c-*fos* [11]. The SAPKs and p38s can also phosphorylate ATF2 at Thr 69 and Thr 71 thereby activating its *trans* activating function [12]. Finally, p38 can phosphorylate (at Thr 293 and 300) and activate the *trans* activating function of myocyte enhancer factor-2C (MEF2C), a transcription factor implicated in stress and cytokine induction of c-*jun* [13]. AP-1 activation by inflammatory cytokines such as TNF and IL1 is crucial to the subsequent induction of additional cytokines, such as IL2 and IL6 as well as additional TNF, and the expression of proteases such as collagenase, which target the extracellular matrix. All of these factors contribute to implementation of the inflammatory response [14-17]. In addition, recent studies have shown that c-Jun and AP-1 activation is critical to apoptosis [18].

The SAPKs contain a characteristic -T_{183}-P-Y_{185}- motif within the L12 loop of subdomain VIII of the catalytic domain [1,4,5]. Phosphorylation of T_{183} and Y_{185} results in SAPK activation and is catalyzed by at least two MEKs, SAPK/ERK kinase-1 (SEK1) and MAPK-kinase (MKK)-7 [19,20]. The PI collaborated with Dr. Leonard Zon to identify SEK1 as a SAPK activator [19]. Similarly, the p38s contain a characteristic phosphorylation loop T-G-Y. Tyr and Thr phosphorylation at these sites results in p38 activation and is catalyzed by at least two MEKs, MKK3 and MKK6 [1,6-8,21,22].

Several protein kinase families have been implicated as MAP3Ks upstream of the SAPKs and p38s. The MEK-kinases (MEKKs) are mammalian homologues of *S. cerevisiae STE11*, a MAP3K that regulates both the mating pheromone and osmosensing pathways [1,2,23]. Mammalian MEKKs include MEKKs-1-4, apoptosis stimulating kinase-1 (ASK1) and TGF-β-activated kinase-1 (TAK1) [24-32]. In collaboration with Dr. Dennis Templeton's laboratory, we showed that MEKK1 could phosphorylate and activate SEK1 [26]. MEKK1 is entirely SAPK specific; and under physiologic circumstances it cannot activate either the mitogenic MAPK pathway or the p38 pathway [1,26]. MEKK4, ASK1 and TAK1 can each activate both the SAPK and p38 pathways *in situ* and can activate SEK1, MKK3 and MKK6 *in vitro* and *in situ* [28-32]. MEKKs-2 and -3 can activate both the SAPK pathway (via SEK1) and the mitogenic MAPK pathway (via MEK1-dependent and -independent mechanisms) [27]. Mixed lineage kinases (MLKs) have also been implicated as MAP3Ks. These kinases bear structural homology to both Ser/Thr and Tyr kinases. MLK2, MLK3 are SAPK-specific while dual lineage kinase (DLK) can activate both the SAPK and p38 pathways [33-35].

The diversity of MAP3Ks upstream of the SAPKs and p38s is a reflection of the diversity of agonists which recruit these pathways. Consistent with this diversity, a broad array of upstream elements feeds into SAPK and p38 core signaling modules. These include the Rho family GTPases Rac1 and Cdc42Hs; constitutively active (GTPase-deficient) Rac1 and Cdc42Hs can activate both the SAPKs and p38s [36-38]. However, the effectors which couple these G proteins to the SAPKs and p38s remain unclear, inasmuch as several upstream activators of the SAPKs and p38s, including MEKK4, MLK3 and kinases of the p21-activated kinase (PAK) family, can bind GTP-charged Rac1 and Cdc42Hs [28,39-41].

1.1.4. *Coupling SAPK and p38 core signaling modules to upstream components: Mammalian Sps1s*

Mammalian homologues of the *S. cerevisiae* protein kinase *SPS1* [42] act independently of Rho family GTPases to recruit the SAPKs. Mammalian Sps1s consist of an amino terminal kinase domain, distantly related to those of the PAKs, and an extended carboxyterminal regulatory domain. The regulatory domains of the different Sps1s are highly divergent [1,42]. The first of the mammalian Sps1s to be identified was germinal center kinase (GCK). GCK is ubiquitously expressed; however, in B follicular tissue, its distribution is restricted to the germinal center. B cell selection and maturation occur in the germinal center and are mediated in part by receptors of the TNFR family including TNFR1, CD40, CD30 and CD27 [43].

Given the segregation of GCK in the germinal center, and the involvement of TNFR1 and CD40, both of which recruit the SAPKs [4,44], in B cell selection, we proposed that GCK might itself be regulated by TNF-like receptors and might couple to the SAPKs. We have since shown that endogenous GCK is activated *in situ* by TNF [45]. Moreover, expression of GCK results in a dramatic activation of the SAPK pathway [45]. The p38s and the MAPKs are not activated [45]. Taken together, these results suggest that GCK is an important effector for coupling TNF receptors to the SAPKs. GCK is constitutively active upon overexpression, an indication that it is regulated by aggregation or by limiting concentrations of an inhibitor, both of which are mechanisms that could be overcome by overexpression. Consistent with a role for the GCK-C-terminus in GCK regulation, we observe that expression of the GCK-C-terminal noncatalytic domain can activate the SAPK pathway, albeit to a much smaller extent than can wild type GCK [45].

Subsequently, two other mammalian Sps1s, hematopoietic progenitor kinase-1 (HPK1) and Nck interacting kinase (NIK) were shown to activate selectively the SAPK pathway [46-48]. HPK1 can interact with both MEKK1 and MLK3, and kinase inactive mutants of these MAP3Ks can inhibit HPK1 activation of the SAPKs. Thus it is likely that MLK3 and MEKK1 are HPK1 targets *in vivo* [46,47]. NIK can also interact with MEKK1; and kinase-dead MEKK1 can block NIK activation of the SAPKs. Thus, it is likely that MEKK1 is a target of NIK [48]. We too see an interaction between MEKK1 and GCK (see section 2.1).

1.1.5 *Signaling to the SAPKs and p38s through the type-1 TNF receptor*

TNF can promote the apoptotic death of numerous cell types, including breast cancer cells [14]. The SAPKs are potently activated by TNF and several recent studies have begun to identify signaling components which couple the TNF receptors (TNFRs) to the SAPKs. TNF is a homotrimeric ligand and TNF binding results in receptor trimerization [49]. A significant advancement in the understanding of TNF signaling came with the discovery of polypeptide species which are recruited to the TNFRs as a result ligand-induced receptor trimerization. Many of these proteins, upon overexpression, can, in a ligand-independent manner, elicit the cellular responses to TNF. The identification of these signal transducers has given rise to the protein recruitment model for TNFR signaling wherein occupancy of the TNFR promotes the binding of signal transducing polypeptides which then relay signals to downstream effectors.

The TNFRs are part of a large family of receptors which share homology within their extracellular domains, but are divergent within their intracellular extensions [49]. Many of these receptors (notably Fas and the type-1 TNF receptor [TNFR1]) contain an ~80 amino acid death domain [50-52]. The death domain is necessary for these receptors to elicit numerous biological responses, including apoptosis. Death domains mediate homotypic and heterotypic protein-protein interactions, thereby coupling receptors to their effectors [50-52]. Thus the death domain-containing polypeptide TNFR associated death domain protein (TRADD) can bind TNFR1 [52].

TRADD can also bind a second polypeptide, TNF receptor-associated factor-2 (TRAF2). The TRADD-TRAF2 interaction serves to recruit TRAF2 to TNFR1 [53-55]. The TRAFs are an emerging class of signal transducers important in TNF family signaling. TRAF polypeptides generally contain carboxyterminal TRAF domains and amino terminal RING and Zn-finger domains. TRAF domains, like death domains, mediate homotypic and heterotypic protein-protein interactions [53-55]. The association between TRADD and TRAF2 requires the C-terminal (amino acids 356-501) of two TRAF domains on TRAF2 and a TRAF binding domain on TRADD (amino acids 106-169) [54,55]. The TRADD-TRAF2 interaction is necessary for coupling TNFR1 to activation of the nuclear factor-κB (NF-κB) transcription factor [52-55]. NF-κB is required for much of the gene expression elicited by TNF [14,51].

Overexpression of TRAF2 not only results in activation of NF-κB, but can also promote potent SAPK and AP-1 activation [56,57], as well as activation of the p38 pathway (see section 2.2). Mutant TRAF2 constructs devoid of the RING finger domain sequester TRADD preventing binding of endogenous TRAF2. By this process, these mutants can inhibit TNF activation of the SAPKs [53,55-57].

Receptor interacting protein (RIP) is a second species which, like TRAF2, can be recruited, in a TNF-dependent manner, to TNFR1 through an interaction with TRADD [58,59]. RIP contains an amino terminal protein kinase domain (amino acids 1-305), an intermediate domain (amino acids 306-553) and a death domain (amino acids 554-656). The latter mediates the TRADD-RIP interaction [58,59]. RIP can also interact with TRAF2 via the RIP intermediate domain and kinase domains and the TRAF2 C-terminal TRAF domain [55,58,59]. RIP overexpression can activate NF-κB and can also activate the SAPK pathway [56,60]. In addition, we have observed activation of the p38 pathway by overexpressed RIP (see section 2.2). Expression of the RIP death domain can block SAPK activation by TNF likely through the sequestration of endogenous TRADD--preventing access to TRADD by either TRAF2 or RIP [56]. The mechanisms by which RIP and TRAF2 couple to the SAPK and p38 pathways are unclear.

1.2 Subject, Purpose of Research

Our interest is to identify and elucidate the biochemistry and cell biology of the SAPK and p38 pathways, signal transduction pathways activated by stress and inflammatory cytokines, and then to characterize these pathways in breast cancer. Our ongoing experiments indicate that these pathways may inhibit cell growth and could thereby counteract the transformation process. In addition, these pathways may actually promote apoptosis [14,18]. Once these pathways are understood, therefore, manipulation of stress signaling at the bedside, through novel therapeutic techniques, could prove efficacious in (1) limiting tumor growth through activation of stress signaling, or, conversely, (2) limiting the toxicity of genotoxic cancer chemotherapeutics by selectively inhibiting their activation of stress pathways.

We have focused this year on three areas of work: 1) Identification of the molecular components which couple GCK to the SAPKs, 2) elucidation of the proximal signaling elements which couple the TNF receptor to the SAPKs and 3) generation of stable cell lines expressing an inducible GCK construct for use in future studies of the cell biology of GCK signaling.

2. BODY

2.1 GCK is a regulated scaffold protein which binds MEKK1

MEKK1 is a mammalian MAP3K whose kinase domain is homologous to that of the *S. cerevisiae* MAP3K *STE11*. The MEKK1 polypeptide is quite large (130-kDa) and consists of a carboxyterminal kinase domain an extensive amino terminal domain [24,25]. Inasmuch as MEKK1 constructs wherein the amino terminus has been totally or partially deleted are constitutively active [24,26], it is likely that the MEKK1 amino terminal domain exerts a negative regulatory effect on MEKK1. The MEKK1 amino terminal region contains several motifs suggestive of complex regulation. These include two plekstrin homology (PH) domains, which may mediate membrane association, and several SH3 binding sites [25]. In addition, MEKK1 contains an acid-rich segment in its amino terminus [24,25] which contains two previously unidentified proline/glutamate/aspartate/serine/threonine-rich (PEST) domains (see below). Both GCK and MEKK1 show a strong preference for activation of the SAPK pathway via activation of SEK1 [1,26,45]. p38 and MAPK are not activated by either except under conditions of massive overexpression [1,26,45]. This observation led us to ask if MEKK1 was a GCK target and if GCK could physically associate with MEKK1. Accordingly, we expressed in 293 cells gluathione-S-transferase (GST)-tagged constructs of wild type GCK or the GCK C-terminal regulatory domain (GCK-CT). The GST polypeptides were isolated on glutathione (GSH) agarose and probed with an antibody to MEKK1 to detect any endogenous MEKK1 bound to the GCK. Fig. 1 shows that endogenous MEKK1 can associate *in situ* with GCK, preferentially interacting with the GCK C-terminus. Binding to full length GST-GCK or GST alone is not detected.

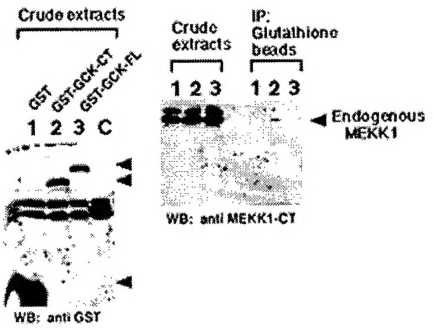

Fig. 1. Interaction between the GCK C-terminal domain and endogenous MEKK1. The numbers indicate the GST constructs transfected into the 293 cells. Left panel: crude extracts blotted with anti GST. Arrowheads indicate, top to bottom, GST-GCK, GST-GCK-CT, GST. Right panel: left three lanes, crude extracts blotted with anti-MEKK1, right three lanes GST isolates blotted with anti MEKK1 antibody. The MEKK1 band is indicated with an arrow. Cells were transfected with the indicated GST-tagged constructs. GSH isolates were subjected to SDS-PAGE and immunoblotting with anti MEKK1 antibody.

We sought next to determine if recombinant MEKK1 displayed a similar preference for the GCK-CT. In addition, we wished to determine the region of the MEKK1 polypeptide necessary for GCK binding. Accordingly, we expressed GST-GCK or GST-GCK-CT with either of three M2-FLAG-tagged MEKK1 constructs: MEKK1 with a partial amino terminal deletion (AAs 817-1493--this deletes both PH domains and the SH3 binding sites [25]), this same construct with part of the catalytic domain (subdomains V-XI) deleted (AAs 817-1340), or all of the catalytic domain deleted (AAs 817-1221) [25]. AAs 817-1221 of MEKK1 forms an acid-/proline-/Ser/Thr-rich domain containing two motifs which obey the criteria of PEST sequences (AAs 1060-1101, PEST score 5.04; AAs 1103-1132, PEST score 2.8 [61]). Fig. 2 shows that all three MEKK1 constructs bind GCK equally well and, like endogenous MEKK1, interact more strongly with the GCK-C-terminal regulatory domain. We do not observe a strong interaction between GCK and other MAP3Ks implicated in SAPK regulation, including ASK1, MLK2 and MLK3.

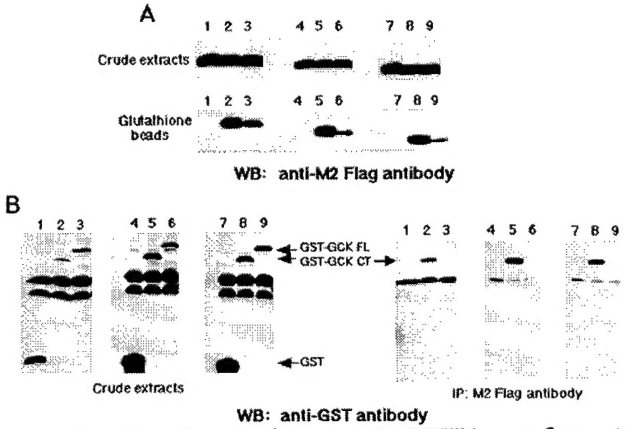

Fig. 2. Recombinant MEKK1 preferentially binds the GCK-CT. The domain on MEKK1 which binds GCK is an acid-/Pro-/Ser/Thr-rich region of the MEKK1 amino terminal regulatory domain (AAs 817-1221). The MEKK1 catalytic domain is not required for binding GCK. 293 cells were transfected with the indicated GST-GCK and M2-FLAG MEKK constructs. (A) Extracts and GSH agarose isolates were probed with anti-M2-FLAG to detect bound MEKK1. (B) Extracts and M2-FLAG immunoprecipitates were probed with anti GST antibody to detect

GCK. Lanes 1-3 transfection with MEKK1 817-1493; lanes 4-6 MEKK1 817-1340); lanes 7-9 MEKK1 817-1221. Lanes 1,4 and 7, GST only; lanes 2, 5 and 8, GST-GCK-CT; lanes 3,6 and 9, GST-full length GCK.

We next wished to map more precisely the region of the GCK-CT that binds MEKK1. The GCK-CT consists of three PEST motifs and a leucine-rich domain [43]. M2-FLAG-tagged constructs wherein these subdomains of the GCK-CT were progressively deleted were expressed in 293 cells with GST-MEKK1. In addition, we assessed the binding of MEKK1 by a kinase-dead (K44M) GCK construct, wherein the Lys residue in the ATP binding domain was mutated to Met. Reciprocal immunoprecipitation/GSH agarose isolations were performed and probed with the cognate antibodies to detect interactions. From Fig. 3, it is clear that the C-terminal most PEST motif (PEST3) is necessary for MEKK1 binding.

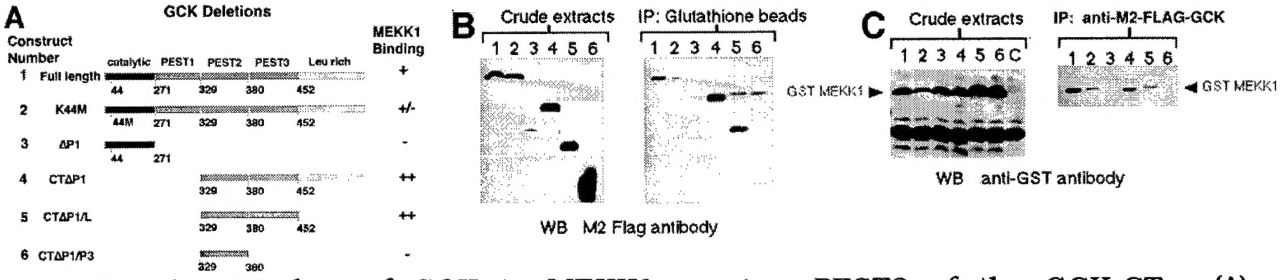

Fig. 3. Binding of GCK to MEKK1 requires PEST3 of the GCK-CT. (A) Schematic illustration of the GCK constructs used. The numbers 1-6 refer to the lanes in (B) and (C). 293 cells were cotransfected with the indicated M2-FLAG-tagged GCK constructs and GST-tagged MEKK1. (B) Extracts or GSH agarose isolates were immunoblotted with anti FLAG to detect GCK. (C) Extracts or FLAG immunoprecipitates were immunoblotted with anti GST antibody to detect MEKK1. Lane labeled C: no transfection.

From Fig. 3, it is also clear that kinase-dead (K44M) GCK, while still able to bind MEKK1 does so even more weakly than does wild type. As before, the GCK-CT binds MEKK1 most strongly. By contrast, we consistently observe that wild type GCK is the most potent SAPK activator, although both K44M-GCK and the GCK-CT can engender substantial SAPK activation [45]. The simplest reconciliation of these results is that activation of GCK's kinase activity promotes MEKK1 binding and mediates a more rapid turnover of MEKK1, thereby allowing for efficient SAPK pathway activation.

In order to demonstrate the functional significance of the GCK-MEKK1 interaction, we performed two experiments. First, we assessed the ability of GCK constructs devoid of PEST3 to activate the SAPKs. Second, we tested the ability of the GCK binding domain of MEKK1 to inhibit GCK activation of coexpressed SAPK. From Fig. 4, it is clear that deletion of PEST3 (ΔP3, Fig. 4), which abrogates completely MEKK1 binding (Fig. 3), also significantly compromises GCK activation of coexpressed SAPK.

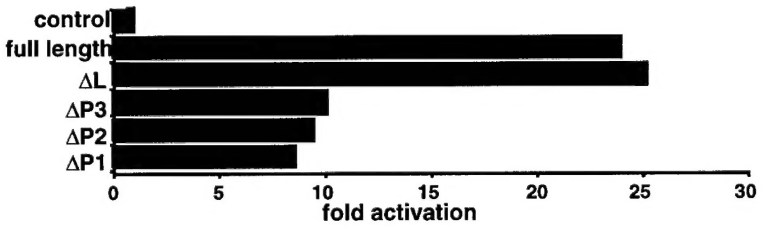

Fig. 4. *In situ* activation of the SAPK pathway by M2-FLAG-GCK deletion constructs. GCK constructs were expressed in 293 cells with HA-SAPK (p46-β1

isoform). SAPKs were subjected to IP and assay for c-Jun kinase activity. P refers to PEST domains, L, the Leu-rich domain.

In addition, expression of MEKK1ΔC (residues 817-1340) can strikingly inhibit GCK activation of coexpressed SAPK (Fig. 5). Inasmuch as this MEKK1 construct binds GCK (Fig. 2), but is devoid of substrate binding domains, does not inhibit GCK's kinase activity (Fig. 5) and cannot bind SEK1 or activate coexpressed SAPK, it is likely that MEKK1ΔC acts by sequestering the expressed GCK and preventing binding of endogenous MEKK1. Once GCK has bound MEKK1, GCK can phosphorylate the GCK binding domain on the MEKK1 polypeptide (Fig. 6). In the experiment shown in Fig. 6, soluble GST-GCK was added to immunoprecipitates of MEKK1 or to blank beads. What is also noteworthy in Fig. 6 is that a significant portion of the GCK added in the experiment is retained on the MEKK1 beads, even after extensive washing. Thus, GCK-MEKK1 complexes can be generated *in vitro.* From the data in Figs. 1-6 we conclude that MEKK1 is a physiologic target of GCK.

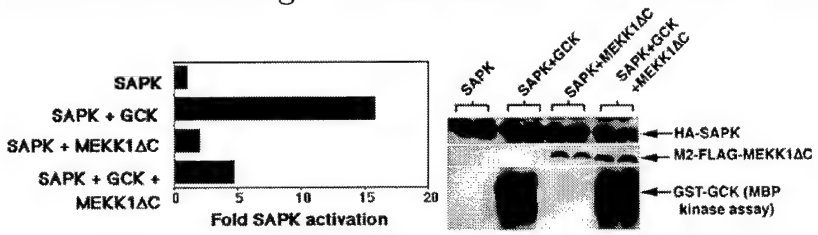

Fig. 5. MEKK1ΔC blocks GCK activation of the SAPKs. 293 cells were transfected with GST-GCK, HA-SAPK or M2-FLAG-MEKK1ΔC as indicated. SAPK was immunoprecipitated and assayed for c-Jun kinase (left panel). Extracts were probed for expression of the indicated constructs (right panel). GCK was assayed for MBP kinase due to the low amount of GCK transfected in this experiment.

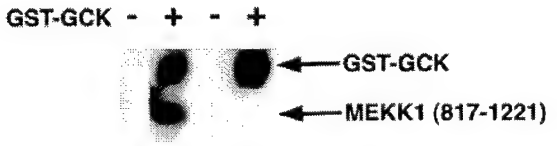

Fig. 6. GCK can phosphorylate the PEST-rich GCK binding domain of MEKK1. 293 cells were transfected with M2-FLAG-MEKK1 (817-1221). GST-GCK was purified from transfected 293 cells by GSH chromatography and elution with free glutathione. MEKK1 (817-1221) was immunoprecipitated and incubated with vehicle or purified GCK and ^{32}P-ATP as indicated. As a control, blank beads (no antibody) were used (far right lane only, all other lanes contained MEKK1 [817-1221]). In the far right lane, the beads were subjected to SDS-PAGE without washing in order to monitor GCK autophosphorylation as a verification of activity. The MEKK1 beads were washed (3 X 1 M LiCl) and subjected to SDS-PAGE. The autophosphorylated GCK and phosphorylated MEKK1 polypeptides are indicated with arrows.

We next sought to determine if GCK was capable of recruiting MEKK1 to the TNFR signaling complex. We have observed that GCK is activated *in situ* by TNF [41]. TNF signaling to the SAPK pathway employs both TRAF2 and RIP [52,53]. We observe a physical association between TRAF2 and the GCK C-terminus (Fig. 7). We do not observe an association between RIP and GCK.

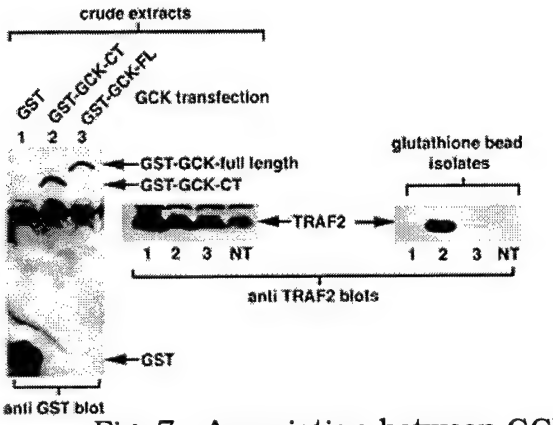

Fig. 7. Association between GCK and TRAF2. 293 cells were transfected with TRAF2 (untagged) and the indicated GST-tagged GCK constructs. GST-tagged polypeptides were purified on GSH agarose and probed in immunoblots with anti TRAF2 antibody. Left seven lanes are crude extracts probed with anti GST antibody or anti TRAF2 antibody as indicated. The right four lanes are an anti TRAF2 blot of GST pulldowns. The numbers refer to the GCK construct transfected; NT: nothing transfected--NT lanes show TRAF2 immunoreactivity due to the presence of endogenous TRAF2 detected by the anti-TRAF2 antibody.

The results in Fig. 7 indicate that GCK can couple to the TNFR signaling complex by associating with TRAF2. Again, the GCK C-terminal tail binds TRAF2 more stably than does full length GCK. As was the case with MEKK1 binding, activation of GCK's kinase activity, possibly as a result of binding TRAF2, might promote the rapid turnover of TRAF2-GCK complexes *in situ*, thereby promoting efficient SAPK pathway activation. The data in Figs. 1-7 demonstrate that by binding both MEKK1 and TRAF2, GCK may act to recruit MEKK1 to the TNFR signaling complex.

2.2 RIP couples both SAPK and p38 to the TNFR signaling complex: p38 activation by RIP may involve a MAP3K associated with the RIP intermediate domain.

Our studies of RIP indicate that it can activate p38 and represents a redundant mechanism for SAPK activation which complements the TRAF2-GCK mechanism described above. Fig. 8 illustrates that the RIP-ID is necessary for activation of both SAPK and p38 by coexpressed RIP; and its deletion abrogates completely the ability of RIP to activate SAPK or p38. The subdomain of the RIP ID which mediates SAPK and p38 activation does not correspond to that (AAs 391-422 [60]) involved in NF-κB activation. The catalytic domain of RIP appears dispensable for SAPK and p38 activation by RIP inasmuch as the catalytic domain itself fails to activate SAPK or p38, and D138N-RIP, which is devoid of catalytic activity [60] can engender robust SAPK and p38 activation.

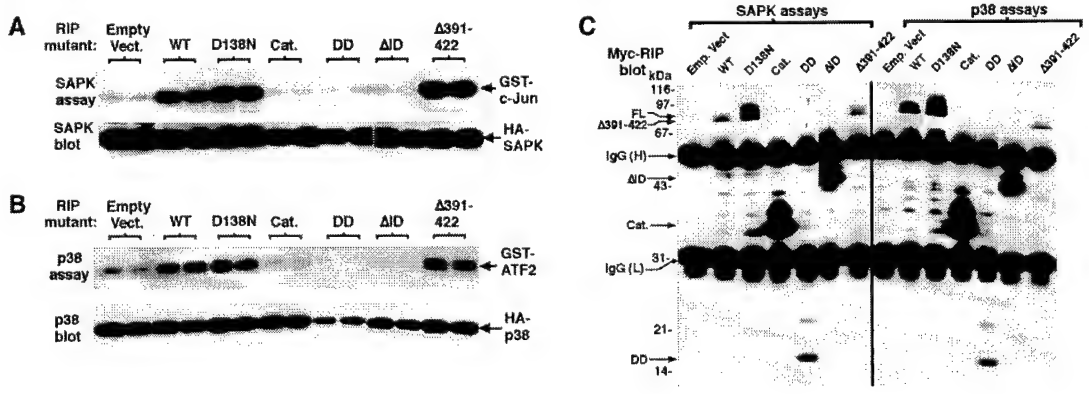

Fig. 8. The RIP ID is necessary for SAPK and p38 activation. The catalytic domain is dispensable. 293 cells were transfected with the indicated RIP constructs. SAPK (A), and p38 (B) were assayed using anti HA immune complexes. Myc-RIP constructs were immunoprecipitated and immunoblotted (C) to assess RIP expression. D138N is a kinase-inactive mutant, Cat, catalytic domain; DD, the RIP death domain; ΔID, deletion of the RIP intermediate domain; Δ391-422, deletion of the ID subdomain implicated [65] in NF-κB activation.

More detailed studies of the isolated RIP-ID indicate that it is sufficient for both SAPK and p38 activation. In addition, our findings indicate that deletion of the RIP-ID (RIP-ΔID) creates a dominant inhibitory molecule that can block SAPK and p38 activation by TNF, likely by sequestering endogenous TRADD (or a related species) which couples directly to TNFR1 [52,58-60] (Fig. 9)

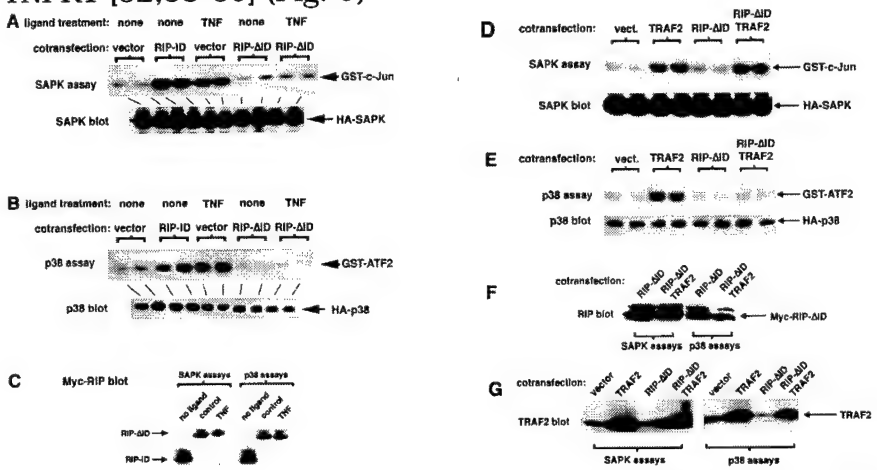

Fig. 9. The RIP ID is necessary and sufficient for RIP activation of the SAPKs. Requirement for the ID for TNF activation of the SAPKs (A-C). D-G show that TRAF2 signals through RIP to activate p38, but not to activate the SAPKs; thus the dominant inhibitory RIP-ΔID mutant blocks TRAF2 activation of p38 but not SAPK.

Fig 9 also shows that RIP is an effector for TRAF2 activation of p38, but not for TRAF2 activation of SAPK. Thus, the dominant inhibitory RIP-ΔID blocks TRAF2 activation of p38 but not of SAPK. The fact that SAPK activation by TRAF2 is not inhibited by RIP-ΔID might be explained by the observation that TRAF2 associates with other species upstream of the SAPKs, such as GCK (Fig. 7).

Our initial studies (see 9/96 report) indicated that RIP could act as a MAP3K. Further study or RIP, combined with our more detailed studies shown in Figs. 8 and 9 have shown that RIP itself is not a MAP3K; and instead suggested to us that RIP might function in a manner analogous to that of GCK--as a binding protein for a MAP3K upstream of the SAPKs, p38s or both. To determine if RIP could associate with a MAP3K, we immunoprecipitated a spectrum of RIP mutants from transfected 293 cells and assayed for activation of the p38-specific MEK, MKK6 *in vitro*. The assay employed the telegraphic format similar to that first designed and used by the PI to assay the mitogenic MAP3K, Raf-1 [62]. Thus, RIP beads were incubated with purified, inactive MKK6 and ATP. A portion of the MKK6 was then removed and used to activate purified, inactive p38. The p38, in turn, was assayed for phosphorylation of ATF2. From the results in Fig. 10, it is evident that the RIP-ID can associate with a MAP3K capable of reconstituting the p38 pathway *in vitro*. The identity of this MAP3K is, at present, unknown. The MAP3K does not correspond to any of the known MAP3Ks upstream of the SAPKs or p38s. We have been unable to detect *in vitro*

activation of SEK1 using this system. Thus RIP may not associate stably with a SAPK-specific MAP3K, or the MAP3K associated with RIP cannot activate SEK1 and, instead targets other SAPK-specific MEKs such as MKK7.

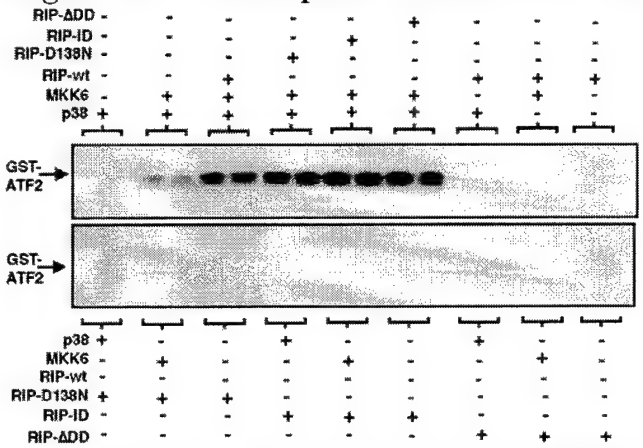

Fig. 10. *In vitro* activation of MKK6 by a MAP3K associated with the RIP-ID. RIP immunoprecipitates or nonimmune IPs were incubated with vehicle or MKK6 plus ATP as indicated. A portion of the MKK6/vehicle was removed and incubated with vehicle or p38 and ATP as indicated. The p38 was then assayed for ATF2 kinase activity. MKK6 and p38 were purified as GST-tagged proteins by GSH-agarose chromatography from extracts of transfected 293 cells, followed by elution with free GSH.

Now that we have clarified some of the signaling elements recruited by TNF which activate the SAPKs, we have begun to incorporate our findings into the realm of breast cancer. To do this, we have generated MCF7/GCKtet, an MCF7 cell line which stably expresses GCK under the control of the TetON system. Fig. 11 shows that these cells will express human hemagglutinin (HA)-tagged GCK upon incubation in doxycycline, a tetracyclin analogue. These cells should allow for the more complete analysis of the cell biology of GCK in breast cancer cells.

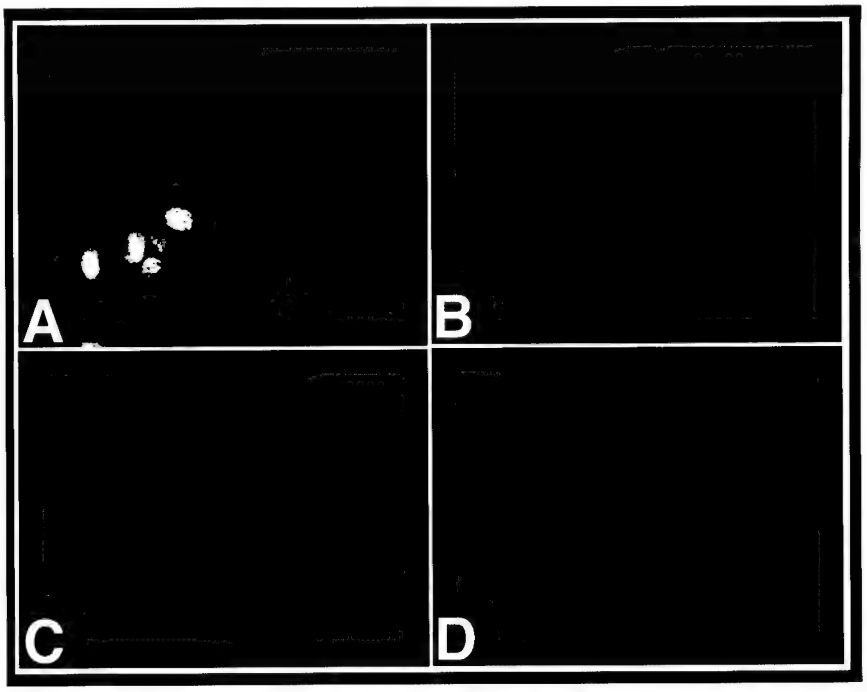

Fig. 11. MCF7/GCKtet, an MCF7 breast cancer cell line which expresses HA-tagged human GCK under a tetracyclin-inducible promoter. A/C: cells incubated in vehicle, B/D cells incubated with 10 μM doxycycline. A/B nuclear staining with Hoechst 33258, C/D detection of induced HA-GCK by staining with a monoclonal antibody to HA and counterstaining with a secondary anti mouse antibody coupled to Texas red.

3. CONCLUSIONS

From the results presented above, we propose a model for TNF signaling which is shown below (Fig. 12). In this model, TRAF2 recruits both GCK and RIP. GCK, in turn, binds and participates in the regulation of MEKK1. MEKK1 activates selectively the SAPKs. RIP retains the potential to activate both the SAPKs and p38s, possibly by associating with a MAP3K upstream of MKK6 and p38, as well as, possibly, MKK7 or other SAPK-specific MEKs. Fig. 12 is a model for TNF signaling which incorporates our findings.

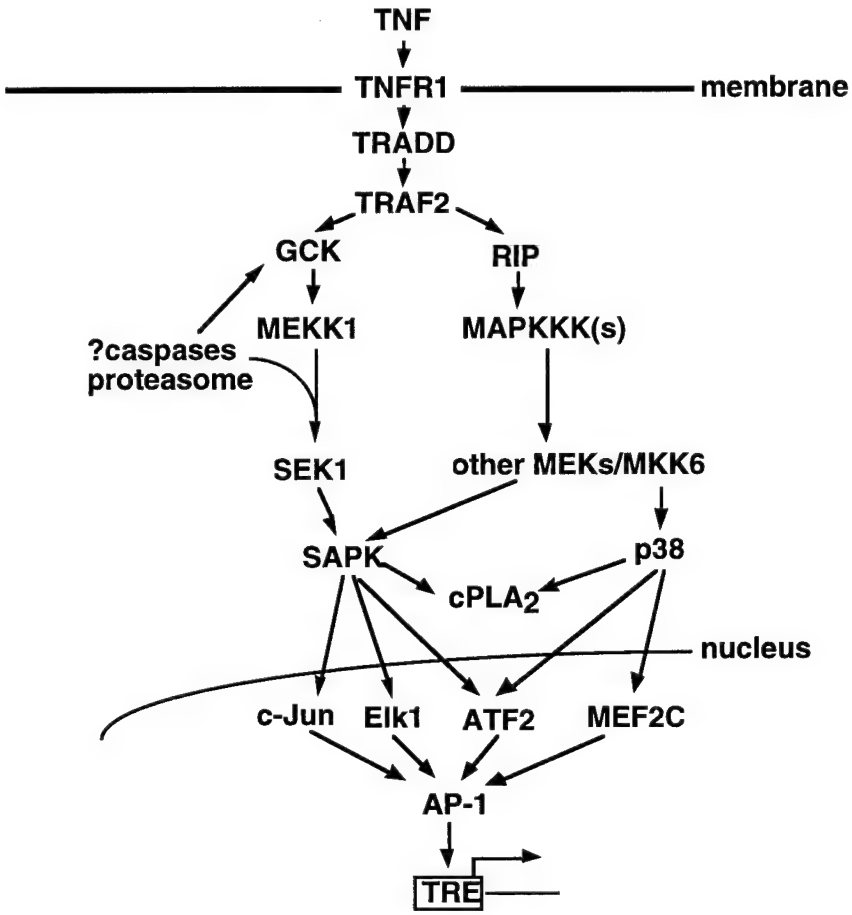

Fig. 12. Model for TNF signaling based on Preliminary Results. TRE is the TPA-response element which binds AP-1, triggering transcription. cPLA2: cytosolic phospholipase-2.

Our studies for the upcoming year will focus on GCK regulation of MEKK1, on identification of the MAP3K associated with RIP and on characterization of MCF7/GCKtet. Specifically:

1) How does GCK regulate MEKK1? We propose that GCK promotes the proteolytic removal of inhibitory sequences within the MEKK1 amino terminal domain.

2) How does TRAF2 regulate GCK? We believe that GCK autoactivates as a consequence of binding TRAF2.

3) What is the identity of the MAPKKK associated with RIP? We propose that RIP associates, directly or indirectly, with a MAPKKK upstream of MKK6 and, possibly, MKK7.

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5. PUBLICATIONS

The following papers have been published/submitted as a result of the project:

1. Molnár, Á., Theodoras, A.M., Zon, L.I., Kyriakis, J.M. (1997) Cdc42Hs, but not Rac1, inhibits serum-stimulated cell cycle progression at G1/S through a mechanism requiring p38/RK. J. Biol. Chem. 272: 13299-13235.

2. Yuasa, T., Liu, H., Pimentel-Muiñoz, F.X., Ting, A., Seed, B., Kehrl, J.H., Kyriakis, J.M. (submitted) Regulation of SAPK and p38 by TNF: germinal center kinase couples TRAF2 to MEKK1 while RIP binds a MAP3K upstream of MKK6.

6. PERSONNEL

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